

***Bacillus amyloliquefaciens* FZB42 represses plant miR846 to induce systemic resistance via a jasmonic acid-dependent signalling pathway**

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SUMMARY

Bacillus amyloliquefaciens FZB42 is a type of plant growth-promoting rhizobacterium (PGPR) which activates induced systemic resistance (ISR) in *Arabidopsis*. Blocking of the synthesis of cyclic lipopeptides and 2,3-butanediol by FZB42, which have been demonstrated to be involved in the priming of ISR, results in the abolishment of the plant defence responses. To further clarify the ISR activated by PGPRs at the microRNA (miRNA) level, small RNA (sRNA) libraries from *Arabidopsis* leaves after root irrigation with FZB42, FZB42 Δ sfp Δ alsS and control were constructed and sequenced. After fold change selection, promoter analysis and target prediction, miR846-5p and miR846-3p from the same precursor were selected as candidate ISR-associated miRNAs. miR846 belongs to the non-conserved miRNAs, specifically exists in *Arabidopsis* and its function in the plant defence response remains unclear. The disease severity of transgenic *Arabidopsis* overexpressing miR846 (OEmiR846) or knockdown miR846 (STTM846) against *Pseudomonas syringae* DC3000 suggests that the miR846 expression level in *Arabidopsis* is negatively correlated with disease resistance. Moreover, miR846 in *Arabidopsis* Col-0 is repressed after methyl jasmonate treatment. In addition, jasmonic acid (JA) signalling-related genes are up-regulated in STTM846, and the stomatal apertures of STTM846 are also less than those in *Arabidopsis* Col-0 after methyl jasmonate treatment. Furthermore, the disease resistance of STTM846 transgenic *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) is blocked by the addition of the JA biosynthetic inhibitor diethylthiocarbamic acid (DIECA). Taken together, our results suggest that *B. amyloliquefaciens* FZB42 inoculation suppresses miR846 expression to induce *Arabidopsis* systemic resistance via a JA-dependent signalling pathway.

Keywords: *Bacillus amyloliquefaciens* FZB42, induced systemic resistance, jasmonic acid, miRNA, sRNA sequence.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPRs) are naturally occurring soil microorganisms that benefit plants by increasing plant growth or reducing disease. The PGPR *Bacillus amyloliquefaciens* FZB42 is an environmental strain that can successfully colonize the roots of plants, such as *Arabidopsis*, stimulate plant growth and enhance the defence response (Chowdhury *et al.*, 2015a; Fan *et al.*, 2012). Analysis of the whole FZB42 genome revealed a potential capability to produce several secondary metabolites, such as lipopeptides (surfactin, iturins, fengycins) and polyketides (macrolactin, bacillaene and difficidin) (Chen *et al.*, 2007). Many secondary metabolites, such as surfactin and 2,3-butanediol, have been confirmed to suppress pathogen growth and to activate the induced systemic resistance process (Cawoy *et al.*, 2014; Ryu *et al.*, 2004).

Plants have developed various mechanisms to protect themselves against bacterial, fungal and viral infection. These defence systems include pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones and Dangl, 2006). The outcome of PTI and ETI can initiate the hypersensitive response (HR) in local tissues and systemic acquired resistance (SAR) in distal tissues (Thomma *et al.*, 2011). SAR is often associated with increased levels of salicylic acid (SA) which coordinate the activation of pathogenesis-related (PR) genes (Fu and Dong, 2013). This systemic resistance can also be activated by beneficial microbes, such as *Pseudomonas* spp. and *Bacillus* spp., which is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009). ISR was discovered by the finding that resistance could be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* wilt of carnation (Van Peer *et al.*, 1991). In many cases, ISR is often dependent on jasmonic acid (JA) and ethylene (ETH) signalling rather than SA signalling in plants. Several studies have shown that volatile organic compounds and cyclic lipopeptides, such as surfactin and fengycin, produced by PGPRs are capable of eliciting ISR in plants (Chowdhury *et al.*, 2015b; Ryu *et al.*, 2004). For example, massetolide A produced by *Pseudomonas fluorescens* SS101 is involved in ISR-eliciting activity in tomato against *Phytophthora infestans* (Tran *et al.*, 2007). Purified

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fengycins and surfactins, but not iturins, induce significant protection in bean and tomato leaves against the fungal pathogen *Botrytis cinerea* (Ongena *et al.*, 2007). Treatment of tobacco cell suspensions with low concentrations of surfactin induces several early plant defence-related events, such as Ca²⁺-dependent extracellular alkalinization and oxidative burst, without causing any significant cell death or any marked toxicity (Jourdan *et al.*, 2009). 2,3-Butanediol released from *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a significantly increases plant resistance against *Pectobacterium carotovorum* ssp. *carotovorum* (Ryu *et al.*, 2004).

MicroRNAs (miRNAs) are short, non-coding, 20–24-nt small RNAs (sRNAs) and have been demonstrated to play an important role in plant immunity. For example, flg22 induces the accumulation of miR393, which contributes to plant resistance against bacteria by negatively regulating the mRNA level of F-box auxin receptors TIR1, AFB2 and AFB3 (Navarro *et al.*, 2006). In addition to miR393, miR167 and miR160, which target auxin-response factors (ARFs), are also induced by *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) *hrcC*–, a strain with a mutation in the type III secretion system (TTSS) that still triggers robust PTI in *Arabidopsis* (Fahlgren *et al.*, 2007). The *Arabidopsis* miRNA-deficient mutants *dcl1* and *hen1* display enhanced growth of the bacterium *Pst* DC3000 *hrcG*, indicating that the proteins required for sRNA biogenesis and function are also required for disease resistance to bacterial pathogens (Navarro *et al.*, 2008). In addition, sRNAs can also trigger plant ETI by regulating resistance (*R*) genes, such as miR482, miR2109 and miR6019/miR6020 (Baldrich and Segundo, 2016; Li *et al.*, 2012). Previous studies have largely focused on miRNAs involved in plant systemic resistance against pathogens. Relatively fewer studies have focused on the role of miRNAs during plant–rhizobacteria interactions. De Luis *et al.* (2012) reported that the legume *Lotus japonicas* miR171c and miR397 are specifically linked to bacterial infection and nodule function rather than organogenesis. In another study, *Bacillus cereus* AR156 pretreatment has been shown to prime ISR to *Pst* DC3000 infection by suppression of miR825 and miR825* and activation of their target toll-interleukin-like receptor-nucleotide-binding site (TIR-NBS) and leucine-rich repeat (LRR)-type *R* genes (Niu *et al.*, 2016). *Bacillus*, *Arabidopsis* and *Pst* DC3000 were introduced into the same research system; however, studies on the interaction between *Bacillus* and plants are needed to explore the miRNAs involved in the ISR process.

In this study, we constructed the *Bacillus amyloliquefaciens* FZB42Δ*sfp*Δ*alsS* mutant, which is deficient in the production of cyclic lipopeptides and 2,3-butanediol compounds. Blocking of the synthesis of these compounds resulted in the abolishment of ISR activation. To identify plant sRNAs involved in the ISR process, we analysed *Arabidopsis* sRNAs by Illumina HiSeq deep sequencing. The results indicated that miR846-5p and miR846-3p were differentially repressed by wild-type FZB42, whereas their expression

showed no significant difference between FZB42Δ*sfp*Δ*alsS* treatment and control. Transgenic *Arabidopsis* overexpressing miR846 was more susceptible to *Pst* DC3000 infection, whereas miR846 knockdown in transgenic *Arabidopsis* showed resistance to *Pst* DC3000. Furthermore, miR846 was confirmed to modulate ISR through the JA-dependent signalling pathway.

RESULTS

Construction of ISR-deficient FZB42Δ*sfp*Δ*alsS* mutant

Cyclic lipopeptides, such as surfactin, and volatile compounds, such as 2,3-butanediol, produced by *Bacillus* have been confirmed to be elicitors of ISR in plants (Chowdhury *et al.*, 2015b; Ryu *et al.*, 2004). The genes *sfp* (encoding phosphopantetheinyl transferase) and *alsS* (encoding acetolactate dehydrogenase) are involved in the synthesis of cyclic lipopeptides and 2,3-butanediol, respectively. In order to prepare an ISR-deficient mutant, we constructed a site-directed mutant of *sfp* and *alsS* genes in FZB42 by double-crossover homologous recombination. The disruption of the *sfp* gene abolishes the production of cyclic lipopeptides, such as surfactin, which has haemolytic activity. The haemolytic activity of the FZB42Δ*sfp*Δ*alsS* mutant disappeared as we expected, and polymerase chain reaction (PCR) amplification also validated that the double mutant constructed was successful (Fig. S1, see Supporting Information).

We then tested the ISR-eliciting activity of the FZB42Δ*sfp*Δ*alsS* mutant through the oxidative burst, stomatal closure, disease severity and disease resistance-related gene expression. FZB42 root inoculation caused *Arabidopsis* stomatal closure and H₂O₂ accumulation, whereas FZB42Δ*sfp*Δ*alsS* root inoculation showed no difference compared with the control (Fig. 1A,B). Moreover, FZB42Δ*sfp*Δ*alsS*-treated *Arabidopsis* showed the same disease severity as the control, and FZB42-treated *Arabidopsis* exhibited an increased disease resistance against *Pst* DC3000 (Fig. 1C). The expression of pathogenesis-related protein (*PR1*), plant defence factor 1.2 (*pdf1.2*) and ETH response factor 1 (*ERF1*) genes, involved in the SA-, JA- and ETH-dependent defence signalling pathways in *Arabidopsis*, were also analysed. The expression of *PR1*, *pdf1.2* and *ERF1* in *Arabidopsis* was enhanced after root inoculation with FZB42 for 12 and 24 h; in contrast their expression levels showed no significant changes in response to FZB42Δ*sfp*Δ*alsS* compared with the control (Fig. 1D). These results indicate that the FZB42Δ*sfp*Δ*alsS* mutant is deficient in ISR activation.

Identification of candidate ISR-associated miRNAs

To identify ISR-associated miRNAs, three sRNA libraries were constructed: *Arabidopsis* leaves after root irrigation with FZB42, FZB42Δ*sfp*Δ*alsS* (mutant deficient in ISR activation) or control. sRNAs ranging from 18 to 30 nucleotides (nt) were isolated from

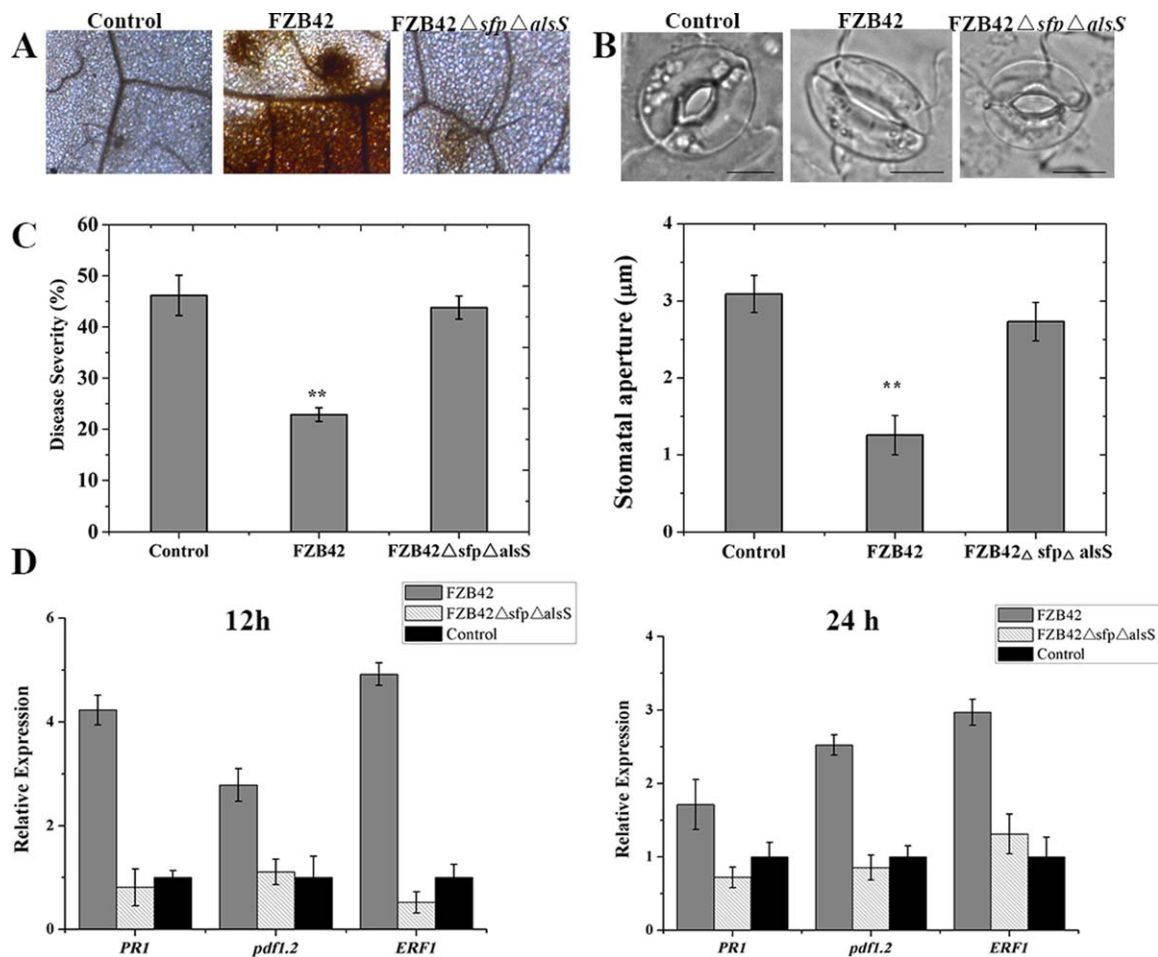


Fig. 1 Hydrogen peroxide (A), stomatal aperture (B), disease severity (C) and expression of disease resistance-related genes (D) in *Arabidopsis* Col-0 leaves after inoculation with *Bacillus amyloliquefaciens* FZB42, the FZB42Δ*sfp*Δ*alsS* mutant and control. (A) Detection of hydrogen peroxide in *Arabidopsis* leaves after inoculation with *B. amyloliquefaciens* FZB42, the FZB42Δ*sfp*Δ*alsS* mutant and control by 3,3'-diaminobenzidine (DAB) staining. Brown precipitates represent hydrogen peroxide generation. (B) Stomatal apertures of *Arabidopsis* leaves after inoculation with *B. amyloliquefaciens* FZB42, the FZB42Δ*sfp*Δ*alsS* mutant and control. Scale bars indicate 10 μm. Values represent the means of 50 random selected stomata. All experiments were repeated three times. **Highly significant difference ($P < 0.01$). (C) Disease severity of *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) after inoculation with *B. amyloliquefaciens* FZB42, the FZB42Δ*sfp*Δ*alsS* mutant and control. Four-week-old *Arabidopsis* Col-0 plants were inoculated with cell suspensions of FZB42, FZB42Δ*sfp*Δ*alsS* and water control. Five days later, leaves were sprayed with a cell suspension of *Pst* DC3000 at 10^8 colony-forming units (CFU)/mL. The disease severity was determined according to the disease index measured at 7 days post-inoculation (dpi). Each treatment had 12 plants and the experiment was repeated three times. (D) Expression levels of disease resistance-related genes in *Arabidopsis* leaves after inoculation with *B. amyloliquefaciens* FZB42, the FZB42Δ*sfp*Δ*alsS* mutant and control for 12 and 24 h. Pathogenesis-related protein (*PR1*), plant defence factor 1.2 (*PDF 1.2*) and ethylene response factor 1 (*ERF1*) genes, involved in the salicylic acid-, jasmonic acid- and ethylene-dependent defence signalling pathways in *Arabidopsis*. Data are expressed as the relative expression of the respective mRNAs normalized to the endogenous actin 2. Error bars represent significant differences according to Fisher's least-significant difference test ($P = 0.05$) using SPSS software. The experiment was repeated three times.

these tissues and sequenced using Illumina. Deep sequencing data revealed 1158k, 1117k and 1141k total reads from the FZB42-, FZB42Δ*sfp*Δ*alsS*- and control-treated *Arabidopsis* leaves, respectively (Table S1, see Supporting Information). In addition, the class of sRNA with a length of 21 nt was the most abundant amongst the three sRNA libraries (Fig. S2, see Supporting Information). Most sRNAs ranging from 18 to 30 nt predominantly favoured a U (uridine) at the 5' end (Fig. S3, see Supporting

Information). Moreover, 252 known miRNAs and 19 novel miRNAs were identified in *Arabidopsis*.

We first screened the differentially expressed miRNAs regarding ISR following the selection criteria Q value < 0.01 and $|\log_2(\text{fold change})| > 1$. Using a two-fold change cut-off, we found that 29 miRNAs were differentially expressed (Table 1). Amongst these differentially expressed miRNAs, three miRNAs were selected for expression analysis using stem-loop quantitative

Table 1 Identified microRNAs (miRNAs) with significant differential expression.

| miR name | miR sequence | log ₂ ^(FZB42/control) | log ₂ ^(FZB42/FZB42ΔsfpΔalsS) | log ₂ ^(FZB42ΔsfpΔalsS/control) |
|--------------|-------------------------|---|--|--|
| miR158a-5p | CUUUGUCUACAUUUUUGGAAA | | -1.6922 | 1.1791 |
| miR159c | UUUGGAUUGAAGGGAGCUCCU | | 1.2171 | 2.2209 |
| miR166e-5p | GGAAUUGUUCUGGCACGAGG | 1.0465 | | |
| miR167d | UGAAGCUGCCAGCAUGAUCUGG | | 1.0657 | |
| miR172a | AGAAUCUUGAUGAUGCUGCAU | -1.3662 | | |
| miR172b-5p | GCAGCACCAUUAAGAUUCAC | | | 1.106 |
| miR172c | AGAAUCUUGAUGAUGCUGCAG | -1.1988 | | |
| miR173-3p | UGAUUCUCUGUGUAAGCGAAA | -2.0867 | -1.4266 | |
| miR1888a | UAAGUUAAGAUUUGAAGAA | -1.7632 | -2.1491 | |
| miR393a-5p | UCCAAAGGGAUUCGCAUUGAUCC | | -1.6796 | |
| miR3932b-5p | UUUGACGUGUCGCAUCUGCUC | | 1.5155 | 1.4638 |
| miR396a-5p | UCCACAGCUUUCUUGAACUG | | 1.0794 | |
| miR398b-3p | UGUGUUCUCAGGUCACCCUG | | | 1.4839 |
| miR399d | UGCCAAAGGAGAUUUGCCCG | | | -1.0753 |
| miR447a.2-3p | UAUGGAAGAAUUGUAGUAUU | | -1.2288 | |
| miR5651 | UUGUGCGGUUCAAAUAGUAAC | -1.2507 | -1.5276 | |
| miR5996 | UGACAUCCAGAUAGAAGCUUUG | -2.0714 | | |
| miR781a | UUAGAGUUUCUGGAUACUUA | -1.5449 | -1.7001 | |
| miR8183 | UUUAGUUGACGGAAUUGUGGC | | 2.3745 | |
| miR824-3p | CCUUCUCAUCG AUGGUCUAGA | 1.0624 | | |
| miR824-5p | UAGACCAUUUGUGAGAAGGGA | | -1.1461 | |
| miR828 | UCUUGCUUAAAUGAGUAUCCA | | | -1.3346 |
| miR840-5p | ACACUGAAGGACCUAAAACUAC | | -1.4847 | |
| miR843 | UUUAGGUCGAGCUUCAUUGGA | | | 1.2199 |
| miR844-3p | UUUAAGCCAUCUUAUCUAGUU | | -1.4455 | |
| miR846-5p | CAUUC AAGGACUUCU AUUCAG | -1.1414 | -1.4858 | |
| miR846-3p | UUGAAUUGAAGUGCUUGAAUU | -1.1029 | -1.0363 | |
| miR850 | UAAGAUCGGACUACAACAAG | 1.2239 | 1.0209 | |
| novel_36 | AUGAAGUGAUGAUUGAACU | -1.4007 | | |

reverse transcription-polymerase chain reaction (qRT-PCR) (Chen *et al.*, 2005). The expression of miR173-3p, miR846-5p and miR846-3p was repressed, consistent with the sRNA sequencing data, which confirmed the reliability of the sRNA sequence (Fig. S4, see Supporting Information). The cross-sections of differentially expressed miRNAs between FZB42 versus FZB42ΔsfpΔalsS and FZB42 versus control were analysed and profiled, because we speculated that ISR-associated miRNAs would be differentially expressed in FZB42-treated *Arabidopsis* versus FZB42ΔsfpΔalsS- and control-treated *Arabidopsis*. According to the criteria, six suppressed miRNAs (miR173-3p, miR1888a, miR5651, miR781a, miR846-5p and miR846-3p) and one induced miRNA (miR850) were identified (Fig. 2A).

Next, the potential defence-responsive elements in the promoters of ISR-associated miRNAs were also investigated. First, the promoters of pri-miRNA were obtained according to Ding *et al.* (2011). *Cis*-acting elements in the miRNA promoters were then searched for by PlantCARE. The TCA element, CGTCA motif and ERE element involved in SA, JA and ETH responsiveness, as well as TC-rich repeats involved in defence responsiveness, were recruited, and their distribution patterns were characterized (Fig. 2B). These defence-related *cis*-acting elements were

present in the promoters of miR173, miR1888, miR781, miR846 and miR850.

We further predicted the putative targets of the above miRNAs by psRobot (Table 2). LRR genes (AT5G01950 and AT5G63020) and jacalin lectin genes (At5G49850 and At5G28520) were predicted as the targets of miR846-5p and miR846-3p, respectively. Interestingly, miR846-5p and miR846-3p originated from the same precursor (Fig. 2C). Thus, miR846-5p and miR846-3p were the most outstanding miRNAs that might play a role in ISR activation. The expression of miR846-5p and miR846-3p was suppressed in FZB42-treated *Arabidopsis*, whereas their expression levels were not altered in FZB42ΔsfpΔalsS- and control-treated *Arabidopsis* (Fig. S4). Jacalin lectin family genes (At5G49850 and At5G28520) were confirmed as the target genes of miR846-3p (Fahlgren *et al.*, 2007; Jia and Rock, 2013a). The expression levels of jacalin-related lectin (JRL) genes (At5G49850 and At5G28520) and LRR genes (At5G01950 and At5G63020) were accordingly induced in FZB42-treated *Arabidopsis* (Fig. 2D). Many JRL genes have been shown to be associated with disease resistance, abiotic stress signalling, wounding insect damage or multiple stresses (Song *et al.*, 2014). Hence, our functional study of ISR-associated miRNAs focused mostly on miR846.

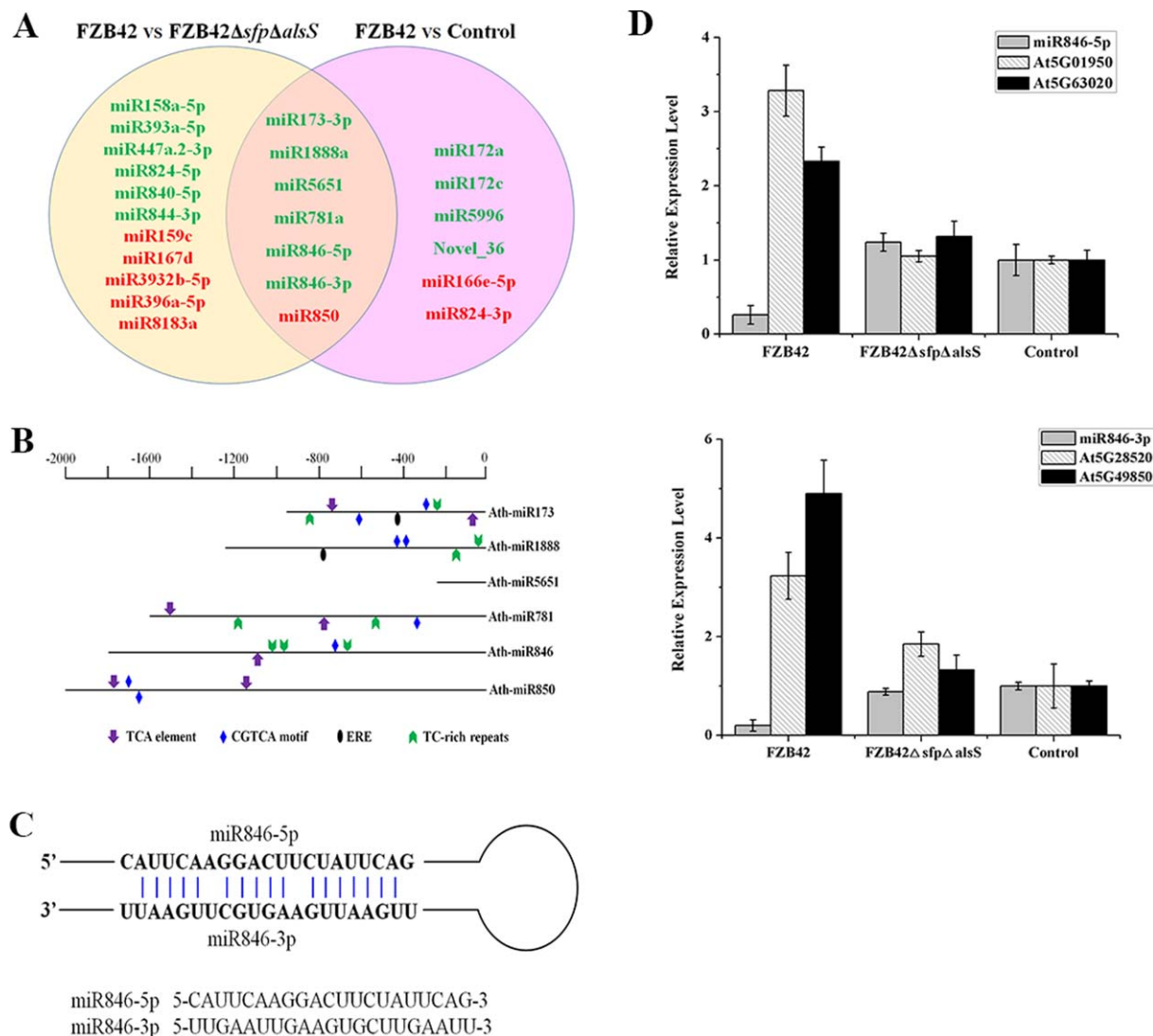


Fig. 2 miR846-5p and miR846-3p are induced systemic resistance (ISR)-associated microRNAs (miRNAs) after inoculation with *Bacillus amyloliquefaciens* FZB42. (A) Venn diagram of differentially expressed miRNAs between FZB42 and FZB42ΔsfpΔalsS and FZB42 and control. Red represents up-regulated miRNAs and green represents down-regulated miRNAs. (B) *Cis*-elements in the promoter regions of differentially expressed miRNAs. The defence-responsive *cis*-elements distributed on the sense strand and reverse strand are shown above and below the black lines, respectively. TCA, CGTCA and ERE elements represent *cis*-acting elements involved in salicylic acid, jasmonic acid and ethylene responsiveness, respectively. TC-rich repeat represents *cis*-acting element involved in defence responsiveness. (C) Secondary stem-loop structure for miR846-5p and miR846-3p with mature miRNA sequences indicated. (D) Expression levels of miR846-5p and miR846-3p and their target genes (At5G01950 and At5G63020 for miR846-5p; At5G49850 and At5G28520 for miR846-3p) from *Arabidopsis* leaves after inoculation with *B. amyloliquefaciens* FZB42, FZB42ΔsfpΔalsS and control. Data are expressed as the relative expression of the respective mRNAs normalized to the endogenous actin 2. The experiment was repeated three times. Error bars represent significant differences according to Fisher's least-significant difference test ($P = 0.05$) using SPSS software.

Expression level of miR846 is negatively correlated with plant resistance ability

To investigate the function of miR846, we generated transgenic *Arabidopsis* overexpressing miR846 (OEmiR846) or with knock-down of miR846 (STTM846). We first investigated the miR846 expression level in OEmiR846 and STTM846 to validate transgenic *Arabidopsis*. The miR846 expression levels in OEmiR846 and

STTM846 were higher and lower, respectively, than in wild-type Col-0, which confirmed the correctness of the constructed OEmiR846 and STTM846 transgenic *Arabidopsis*. In addition, the expression of target genes (At5G49850 and At5G28520 for miR846-3p; At5G01950 and At5G63020 for miR846-5p) was repressed in OEmiR846 transgenic *Arabidopsis*, and the target genes were up-regulated in STTM846 transgenic *Arabidopsis*

Table 2 Predicted targets of microRNAs (miRNAs) in *Arabidopsis*.

| miR name | Accession no. of miRNA targets | Protein annotation |
|-----------|--------------------------------|---|
| miR173-3p | AT1G53600 | Pentatricopeptide repeat-containing protein |
| | AT4G38960 | B-box type zinc finger family protein |
| miR1888a | AT1G18090 | 5'–3' exonuclease family protein |
| | AT1G28620 | Pseudo |
| | AT1G35740 | Pseudo |
| | AT1G53630 | Pseudo |
| miR781a | AT1G44900 | Minichromosome maintenance protein 2 |
| | AT1G52820 | 2-Oxoglutarate-dependent dioxygenase |
| | AT1G69490 | NAC transcription factor protein family |
| | AT2G37760 | Aldo–keto reductase family 4 member C8 |
| | AT5G23480 | SWIB/MDM2, Plus-3 and GYF domain-containing protein |
| miR846-5p | AT5G01950 | Leucine-rich repeat protein kinase-like protein |
| | AT5G63020 | CC-NBS-LRR class disease resistance protein |
| miR846-3p | AT1G33790 | Jacalin lectin family protein |
| | AT1G52050 | Jacalin-like lectin domain-containing protein |
| | AT1G52060 | Jacalin-like lectin domain-containing protein |
| | AT1G52070 | Jacalin-like lectin domain-containing protein |
| | AT1G52110 | Jacalin-like lectin domain-containing protein |
| | AT1G52130 | Mannose-binding lectin-like protein |
| | AT1G57570 | Jacalin-like lectin domain-containing protein |
| | AT1G60095 | Jacalin-like lectin domain-containing protein |
| | AT1G60110 | Jacalin-like lectin domain-containing protein |
| | AT1G61230 | Jacalin-like lectin domain-containing protein |
| | AT2G25980 | Myrosinase-binding protein-like protein |
| | AT2G42270 | U5 small nuclear ribonucleoprotein helicase |
| | AT3G60730 | Putative pectinesterase/pectinesterase inhibitor 36 |
| | AT5G28520 | Mannose-binding lectin-like protein |
| | AT5G38540 | Mannose-binding lectin superfamily protein |
| | AT5G38550 | Jacalin lectin family protein |
| | AT5G49850 | Jacalin lectin family protein |
| | AT5G49870 | Mannose-binding lectin superfamily protein |
| miR850 | AT3G28007 | Nodulin MtN3 family protein |
| | AT2G43240 | Nucleotide-sugar transporter family protein |

CC-NBS-LRR, coiled coil-nucleotide-binding site-leucine-rich repeat.

(Fig. 3A). STTM846 transgenic *Arabidopsis* had a smaller stature than the wild-type, whereas OEmiR846 transgenic *Arabidopsis* had a larger stature (Fig. 3B). We further detected the disease resistance of transgenic *Arabidopsis* against *Pst* DC3000. The disease severity and *Pst* DC3000 density in OEmiR846 were significantly higher than those in the wild-type. In contrast, the disease severity and *Pst* DC3000 density in STTM846 were less than those in the wild-type (Fig. 3C,D). Evans blue staining showed similar results (Fig. 3E). We also detected the expression levels of disease resistance-related genes in OEmiR846 and STTM846. The disease resistance-related gene *pdf1.2* was significantly induced in STTM846 (Fig. 3F). Taken together, these results indicate that the expression level of miR846 in *Arabidopsis* is negatively correlated with plant disease resistance.

miR846 exerts its regulatory function in ISR through JA-dependent signalling in *Arabidopsis*

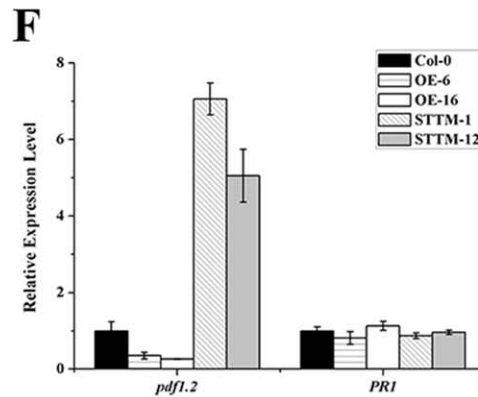
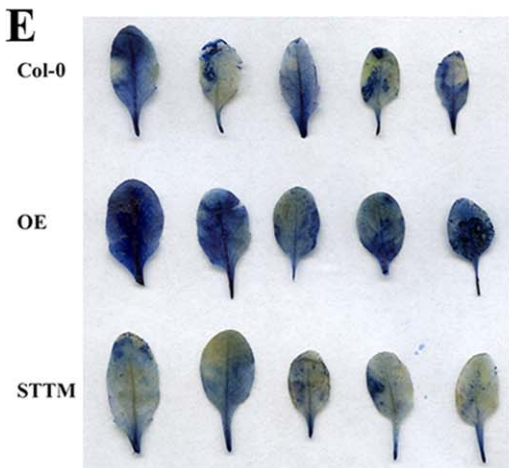
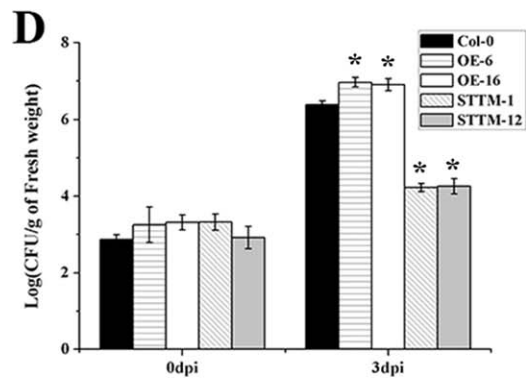
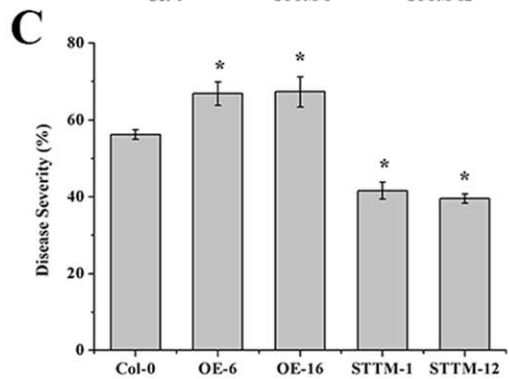
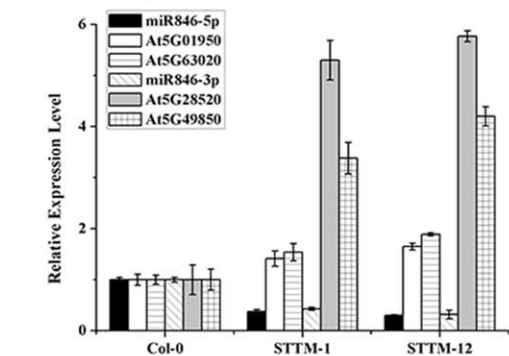
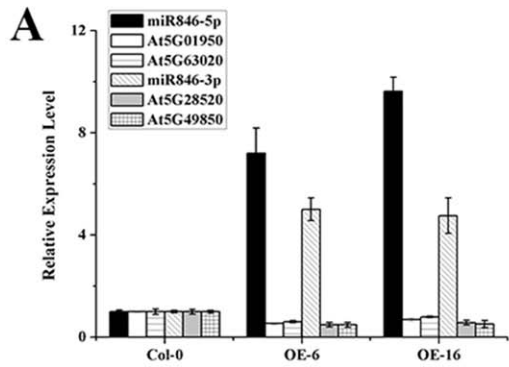
SAR primed by pathogens is often dependent on the SA signalling pathway, and many miRNAs have been confirmed to play a role in SAR, such as miR393 and miR482 (González *et al.*, 2015; Navarro *et al.*, 2006). ISR elicited by beneficial bacteria is often dependent on the JA signalling pathway; however, the function of miRNA in the ISR-activating process remains unclear. The up-regulated expression of *pdf1.2* in STTM846 suggests that miR846 may be involved in the JA signalling pathway. To further confirm this, we first detected the miR846 expression level in wild-type *Arabidopsis* Col-0 after spraying with SA and methyl jasmonate (MeJA). miR846-5p and miR846-3p were both repressed by spraying with MeJA, as expected (Fig. 4A). In addition, the expression levels of *AOS*, *LOX2* and *LOX3*, associated with JA biosynthesis, in OEmiR846 and STTM846 were also observed. The three JA biosynthesis-related genes were all up-regulated in STTM846 (Fig. 4B). The stomatal apertures of OEmiR846 and STTM846 transgenic *Arabidopsis* showed no difference under light conditions. However, the addition of MeJA led to stomatal closure in STTM846, and the stomatal apertures of OEmiR846 were larger than those of wild-type Col-0 (Fig. 4C). Furthermore, the disease resistance of STTM846 transgenic *Arabidopsis* against *Pst* DC3000 was blocked by spraying with the JA biosynthetic inhibitor diethyl-diethiocarbamic acid (DIECA) (Fig. 4D). All of these results suggest that miR846 modulates ISR through the JA-dependent signalling pathway.

DISCUSSION

Numerous reports have demonstrated that plant sRNAs are involved in the evolution of plant immune systems in response to pathogens (Padmanabhan *et al.*, 2009). However, whether miRNAs contribute to the ISR-eliciting process in response to biocontrol bacteria still remains unclear. In the present study, we successfully constructed an ISR-deficient mutant of *B. amyloliquefaciens* (FZB42 Δ *sfp* Δ *als5*) by blocking cyclic lipopeptide and 2,3-butanediol synthesis. This provides the foundation for the analysis of the *Arabidopsis* sRNA profile in response to FZB42 (triggers ISR), FZB42 Δ *sfp* Δ *als5* (deficient in ISR) and control to identify miRNAs involved in the ISR process. After fold change selection, promoter analysis and target prediction, miR846-5p and miR846-3p from the same precursor were selected as candidate ISR-associated miRNAs. miR846 is only present in *Arabidopsis*, and belongs to the non-conserved miRNAs. Non-conserved miRNAs can be distinguished from conserved miRNAs by the feature of existing only in limited plant species (Qin *et al.*, 2014). Several non-conserved miRNAs have been confirmed to be involved in plant defence responses. For instance, tomato miR482 and tobacco miR6019 are typical species-specific miRNAs that modulate plant immunity by cleaving LRR disease resistance (Li *et al.*, 2012). Another non-conserved miR472

also influences PTI via the mediation of secondary siRNAs (Boccaro *et al.*, 2014). These observations show the significant importance of non-conserved miRNAs in plant defence, and that different types of

plant may adopt different strategies at the sRNA level to elicit resistance against various microbes, including pathogens and PGPRs, during their interaction process. miR846 was first reported



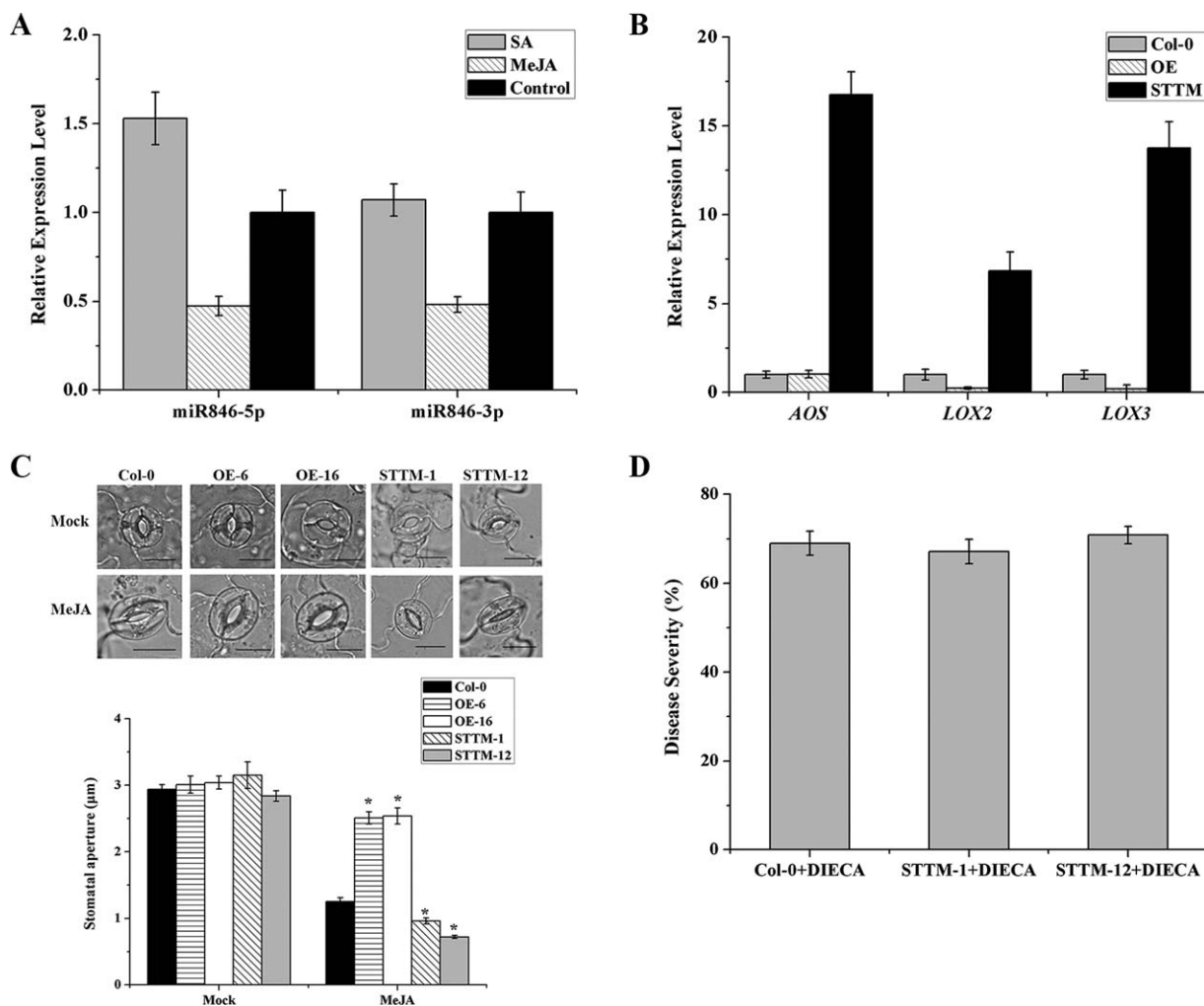


Fig. 4 miR846 modulates induced systemic resistance in *Arabidopsis* through the jasmonic acid (JA)-dependent signalling pathway. (A) Expression levels of miR846-5p/miR846-3p were detected in wild *Arabidopsis* Col-0 after spraying with 1 mM salicylic acid (SA) or 0.1 mM methyl jasmonate (MeJA). (B) Expression levels of JA biosynthesis genes (*AOS*, *LOX2* and *LOX3*) in transgenic *Arabidopsis* OEmiR846 and STTM846. (C) Stomatal apertures in transgenic *Arabidopsis* OEmiR846 and STTM846 after the addition of 0.1 mM MeJA. Scale bars indicate 10 μ m. Values represent the means of 50 random selected stomata. (D) Disease resistance of STTM846 against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) after spraying with the JA biosynthetic inhibitor diethylthiocarbamic acid (DIECA). Four-week-old STTM846 transgenic *Arabidopsis* and wild-type Col-0 were previously sprayed with 200 μ M DIECA containing 0.02% (v/v) Tween-20. Twenty-four hours later, DIECA-treated plants were inoculated with 10⁸ colony-forming units (CFU)/mL *Pst* DC3000. Disease severity was determined according to the disease index measured at 7 days post-inoculation (dpi). Each treatment had 12 plants and the experiment was repeated three times. Error bars represent significant differences according to Fisher's least-significant difference test ($P = 0.05$) using SPSS software. *Highly significant difference ($P < 0.05$). All experiments were repeated three times.

Fig. 3 The miR846 expression level in *Arabidopsis* is negatively correlated with disease resistance. (A) Expression levels of miR846-5p/miR846-3p and their target genes were detected in transgenic *Arabidopsis* overexpressing miR846-5p/miR846-3p (OEmiR846) or with knockdown of miR846-5p/miR846-3p (STTM846) by stem-loop quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The leucine-rich repeat genes (AT5G01950 and AT5G63020) were predicted as the targets of miR846-5p. The jacalin lectin genes (At5G49850 and At5G28520) were predicted as the targets for miR846-3p. (B) Phenotypes of transgenic *Arabidopsis* OEmiR846 and STTM846. Photographs were taken 4 weeks after germination. (C) Disease severity of transgenic *Arabidopsis* OEmiR846 and STTM846 after *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection for 7 days. *Highly significant difference ($P < 0.05$). (D) Quantification of *Pst* DC3000 growth in transgenic *Arabidopsis* OEmiR846 and STTM846. *Highly significant difference ($P < 0.05$). CFU, colony-forming unit; dpi, days post-inoculation. (E) Cell death in transgenic *Arabidopsis* OEmiR846 and STTM846 leaves after *Pst* DC3000 infection for 7 days. Blue areas represent cell death. (F) Expression levels of disease resistance-associated genes in transgenic *Arabidopsis* OEmiR846 and STTM846 were detected by qRT-PCR. Pathogenesis-related protein (*PR1*) and plant defence factor 1.2 (*PDF 1.2*) represent marker genes involved in the salicylic acid- and jasmonic acid-dependent defence signalling pathways in *Arabidopsis*. Error bars represent significant differences according to Fisher's least-significant difference test ($P = 0.05$) using SPSS software. Each experiment was repeated three times.

in the miRNA diversity analysis of *Arabidopsis thaliana* (Rajagopalan *et al.*, 2006). In addition, miR846 has also been mentioned in several reports. For example, the expression of miR846 was repressed by the application of exogenous abscisic acid (ABA) to *Arabidopsis* seedlings, whereas miR846 was dramatically up-regulated under nitrogen starvation conditions (Jia and Rock, 2013b; Liang *et al.*, 2012). In addition, miR846 was also induced in transgenic *Arabidopsis* overexpressing *rGRF3* or purple acid phosphatase 2 (Liang *et al.*, 2014; Liu *et al.*, 2014). However, these studies only reported the expression level of miR846 under different conditions; the function of miR846, especially in plant defence responses, remains unknown. miR846 was predicted to target several jacalin lectin genes; two jacalin lectin genes (At5G49850 and At5G28520) have been confirmed as the targets of miR846 through 5' RNA ligase-mediated rapid amplification of cDNA ends (5'RLM-RACE) (Fahlgren *et al.*, 2007; Jia and Rock, 2013a). The family of JRL genes is named after jacalin, an 18-kDa T-antigen disaccharide-binding lectin domain first isolated from the seeds of jackfruit (*Artocarpus integrifolia*). Many JRL genes have been shown to be associated with disease resistance, abiotic stress signalling, wounding insect damage or multiple stresses (Song *et al.*, 2014). Transgenic tobacco plants overexpressing the JRL gene Ta-JA1 show increased resistance to bacterial, fungal and viral pathogens (Ma *et al.*, 2010). *Arabidopsis* jacalin-related JAX1 confers broad but specific resistance to potex viruses by the inhibition of viral RNA accumulation (Yamaji *et al.*, 2012). Hence, we predicted that *B. amyloliquefaciens* FZB42 activated *Arabidopsis* ISR by the suppression of miR846.

To further validate our hypothesis, we constructed transgenic *Arabidopsis* overexpressing OEmiR846 and the knockdown mutant STTM846. The expression level of miR846 in *Arabidopsis* was negatively correlated with plant disease resistance, indicating that miR846 negatively modulates ISR. On the other hand, jacalin lectins, the target genes of miR846, can be subdivided into two subgroups (galactose-specific JRLs and mannose-specific JRLs) based on differences in molecular structure, subcellular localization and carbohydrate-binding properties. A mannose-specific JRL TaJRL1, consisting of two jacalin-like domains, is considered to be a component of SA- and JA-dependent plant defence signalling. *A. thaliana* transformed with TaJRL1 displays increased resistance to *Fusarium graminearum* and *Botrytis cinerea* (Xiang *et al.*, 2011). The phytohormones SA, JA and ETH play important roles in plant defence responses. ISR is often regulated by JA/ETH-dependent signalling pathways. To understand the signalling pathway in which miR846 is involved, the miR846 expression level in wild-type *Arabidopsis* Col-0 in response to SA and MeJA, as well as the expression levels of JA biosynthesis-related genes in transgenic OEmiR846 and STTM846, were detected. Our results suggest that miR846 may negatively regulate ISR via the JA-dependent signalling pathway.

Previous studies investigating the functions of miRNAs in plant defence have focused on SAR elicited by pathogens. The involvement of miRNAs in beneficial bacteria-primed ISR processes has been ignored. Our study has revealed that lipopeptides and volatile compounds produced by *B. amyloliquefaciens* FZB42 repress *Arabidopsis* miR846 to induce defence-related gene expression and stomatal closure, thus leading to ISR. Moreover, miR846 modulates ISR via the JA-dependent signalling pathway.

EXPERIMENTAL PROCEDURES

Bacterial strains, plant and growth conditions

Bacillus amyloliquefaciens FZB42 wild-type and FZB42 Δ *sfp* mutant were provided by Professor Gao from Nanjing Agricultural University, Nanjing, China. *Bacillus amyloliquefaciens* FZB42 wild-type and FZB42 Δ *sfp* Δ *alsS* were grown in Luria–Bertani (LB) medium at 37 °C for 12 h. The antibiotics 5 µg/ml chloramphenicol and 10 µg/ml erythromycin were added to culture FZB42 Δ *sfp* Δ *alsS*. Subsequently, bacterial cells were harvested by centrifugation, resuspended in distilled water and adjusted to a final concentration of 10⁸ colony-forming units (CFU)/mL.

Pseudomonas syringae pv. *tomato* DC3000 was grown in King's B (KB) medium at 28 °C for 24 h. Cells were then collected by centrifugation and resuspended in 10 mM MgSO₄ containing 0.01% (v/v) surfactant Silwet L-77 with a final concentration of 10⁸ CFU/mL.

Arabidopsis thaliana Col-0 seeds were surface sterilized with 0.12% NaClO for 15 min, washed three times with sterile distilled water, spread on solid Murashige and Skoog (MS) medium and vernalized for 3 days at 4 °C in the absence of light. Seedlings were then placed in growth cabinets under a 16-h/8-h light/dark condition at 22 °C.

Construction of mutants

To generate the site-directed mutant FZB42 Δ *sfp* Δ *alsS*, two partial sequence fragments of *alsS* genes were amplified from FZB42 chromosomal DNA; the sequences of chloramphenicol were obtained from plasmid pAD43-25. The recombinant fragments were fused by overlap extension PCR and transformed into the FZB42 Δ *sfp* strain to generate the site-directed mutant FZB42 Δ *sfp* Δ *alsS*.

To generate the overexpression construct OEmiR846, the miR846 precursor sequence was cloned from the *A. thaliana* genome, and then inserted into the plant overexpression vector pCambia1301. Short tandem target mimic (STTM) was used to inactivate miR846 (Yan *et al.*, 2012). The STTM846 construct carries two tandem miR846 binding sites linked by a 48-nt spacer. The miR846 binding site contains perfect complementary sequences of miR846, with the exception of the 'CTA' bulges, to prevent miR846-mediated cleavage. The construct was then inserted into the vector pCambia1301. The constructs OEmiR846 and STTM846 were electroporated into *Agrobacterium tumefaciens* GV3101, and used to transform *Arabidopsis* by the floral dipping method.

Reactive oxygen species (ROS) assay

Arabidopsis leaves collected 6 h after treatment were incubated in phosphate-buffered saline (PBS) containing 1 mg/mL 3,3'-diaminobenzidine

(DAB) for 8 h. The leaves were then boiled in 95% ethanol for 15 min, and brown precipitates were observed (Samuel *et al.*, 2005).

Stomatal aperture measurement

Arabidopsis Col-0 leaf epidermis was excised from *Bacillus*-inoculated plants after 5 h of exposure to light within the growth chamber and floated in MES buffer (10 mM MES-Tris, 5 mM KCl, and 50 mM CaCl₂, pH 6.15). The leaf epidermis from transgenic *Arabidopsis* leaves was first soaked in MES buffer under light for 6 h to open the stomata, followed by the addition of 0.1 mM MeJA for 4 h. The stomatal aperture was detected using a Leica microscope (Wetzlar, Germany), and the diameters were measured from 50 randomly selected stomata using ImageJ software. Each assay was repeated three times.

Cell death measurement

Cell death was measured by Evans blue staining. *Arabidopsis* leaves which had been inoculated with *Pst* DC3000 for 7 days were soaked in 0.5% Evans blue for 24 h, and then soaked in 1 g/mL chloral hydrate for 24 h. The blue areas representing cell death were observed.

Induction of systemic resistance

The roots of 4-week-old *Arabidopsis* were inoculated with a cell suspension of FZB42, FZB42Δ*sfp*Δ*alsS* or water control. Five days later, *Arabidopsis* was challenge inoculated by spraying the leaves with *Pst* DC3000 at 10⁸ CFU/mL and then kept in a dew chamber at 100% relative humidity for 3 days. After challenge inoculation for 7 days, the disease index was assessed according to Hossain and associates (Hossain *et al.*, 2007). Each treatment had 12 plants and the experiment was repeated three times.

sRNA library construction and deep sequencing

After 12 h of root inoculation with FZB42, FZB42Δ*sfp*Δ*alsS* or control, 24 *Arabidopsis* leaves per sample were collected. sRNA extraction and library construction were carried out by Novogene (Beijing, China). A total amount of 3 μg of total RNA per sample was used as input material for the sRNA library. Sequencing libraries were generated using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, Ipswich, MA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. sRNAs (18–30 nt) were excised and ligated to 5'- and 3'-RNA adaptors using T4 RNA ligase, followed by RT-PCR and gel purification, as instructed by Illumina. The sRNA libraries were sequenced on an Illumina HiSeq 2500/2000 platform.

Sequence data processing

Raw data in fastq format were first processed through custom perl and python scripts; clean data were then obtained by removing reads containing poly-N, with 5' adapter contaminants, without 3' adapter or the insert tag, containing poly A or T or G or C, and low-quality reads from raw data. Meanwhile, the sRNA sequences were mapped to the genome using *A. thaliana* without mismatch to analyse their expression and distribution on the reference. *P* values were adjusted using the *Q* value. *Q* value < 0.01 and |log₂(fold change)| > 1 was set as the threshold for

significantly differential expression by default. Prediction of the target gene of miRNA was performed by psRobot_tar in psRobot for plants (Wu *et al.*, 2012).

miRNA promoter selection and cis-acting element analysis

The promoter sequences of pri-miRNAs were obtained as follows (Ding *et al.*, 2011). In general, if pri-miRNA and its closest upstream gene had the same transcription, and the distance between them was less than 2400 bp, the region from the site 400 bp downstream of the upstream gene to pri-miRNA was obtained. If the pri-miRNA and its upstream gene had the opposite transcription, and their distance was less than 4000 bp, the region between pri-miRNA and their middle point was used. Otherwise, the 2000-bp upstream area of pri-miRNA was used. PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was then used to analyse these miRNA promoters. Cis-acting elements involved in SA, MeJA, ETH and defence responsiveness were retrieved.

qRT-PCR

Stem-loop qRT-PCR was used to measure the expression of miRNA. Total RNA was extracted from *Arabidopsis* leaves using TRIZOL reagent. The reverse transcription reaction was performed according to a PrimeScript™ RT kit (TaKaRa, Dalian, China) with stem-loop reverse transcription primer. Stem-loop qRT-PCR was then carried out on an ABI 7300 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used as the internal control.

Statistical analysis

Three independent experiments were performed for each assay. The data were analysed by Fisher's least-significant difference test (*P* < 0.05) with SPSS software (SPSS Inc. Chicago, USA).

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COMPETING INTERESTS

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Construction of the induced systemic resistance (ISR)-deficient mutant FZB42 Δ sfp Δ alsS. (A) Haemolytic activity detection of the FZB42 Δ sfp Δ alsS mutant: 1, FZB42; 2, FZB42 Δ sfp Δ alsS mutant; 3, methyl alcohol control. (B) Polymerase chain reaction (PCR) amplification verification of the FZB42 Δ sfp Δ alsS mutant: 1, FZB42; 2, FZB42 Δ sfp mutant; 3–5, FZB42 Δ sfp Δ alsS transformants; 6, sterilized water control.

Fig. S2 Length distribution of small RNAs from the sequencing of the three *Arabidopsis* libraries. (A) *Arabidopsis* leaves treated by wide-type FZB42. (B) *Arabidopsis* leaves treated by mutant FZB42 Δ sfp Δ alsS. (C) *Arabidopsis* leaves treated by control.

Fig. S3 Relative nucleotide bias at the first position of the known *Arabidopsis* microRNAs (miRNAs). (A) *Arabidopsis* leaves treated by wide-type FZB42. (B) *Arabidopsis* leaves treated by mutant FZB42 Δ *sfp* Δ *alsS*. (C) *Arabidopsis* leaves treated by control.

Fig. S4 The expression levels of three differentially expressed microRNAs (miRNAs) were examined by stem-loop quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Table S1 Summary of small RNA (sRNA) sequencing.